Mechanisms of the Genotoxicity of Crocidolite Asbestos in Mammalian Cells: Implication from Mutation Patterns Induced by Reactive Oxygen Species

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Asbestos is an important environmental carcinogen in the United States and remains the primary occupational concern in many developing countries; however, the underlying mechanisms of its genotoxicity are not known. We showed previously that asbestos is a potent gene and chromosomal mutagen in mammalian cells and that it induces mostly multilocus deletions. Furthermore, reactive oxygen species (ROS) are associated with the mutagenic process. To evaluate the contribution of ROS to the mutagenicity of asbestos, we examined their generation, particularly hydrogen peroxide, and compared the types of mutants induced by crocidolite fibers with those generated by H_2O_2 in human–hamster hybrid (A_L) cells. Using confocal scanning microscopy together with the radical probe 5′,6′-chloromethy-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA), we found that asbestos induces a dose-dependent increase in the level of ROS among fiber-treated A_L cells, which is suppressed by concurrent treatment with dimethyl sulfoxide. Using N-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent) together with horseradish peroxidase, we further demonstrated that there was a dose-dependent induction of H_2O_2 in crocidolite-treated A_L cells. The amount of H_2O_2 induced by asbestos reached a plateau at a dose of 6 μg/cm^2. Concurrent treatment with catalase (1,000 U/mL) inhibited this induction by 7- to 8-fold. Mutation spectrum analysis showed that the types of *CD59*$^-$ mutants induced by crocidolite fibers were similar to those induced by equitoxic doses of H_2O_2. These results provide direct evidence that the mutagenicity of asbestos is mediated by ROS in mammalian cells. *Key words:* 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent), 5′,6′-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, crocidolite asbestos, human–hamster hybrid cell, hydrogen peroxide, mutation spectrum, reactive oxygen species. *Environ Health Perspect* 110:1003–1008 (2002). [Online 16 August 2002]
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Asbestos fibers are fibrous mineral silicates that have been associated with the development of pulmonary fibrosis, bronchogenic carcinoma, and malignant mesotheliomas in both humans and experimental animals (*1,2*). The fact that asbestos, a well-established carcinogen, has been used extensively in industry and households makes it an important health concern. It has been suggested that the danger of developing asbestos-related diseases extends beyond that of a simple occupational hazard because it has been documented in family members of asbestos workers, in individuals living in the neighborhood of industrial sources of asbestos, and in some schools and public buildings where asbestos is being used as insulation material (*3–5*). Moreover, resuspension of materials from asbestos-containing ceilings has been shown to be the main source of asbestos pollution in old, poorly maintained buildings (*6*). The continued discovery of routes through which the general public may be exposed to asbestos suggests a long-term, low-dose exposure of a large number of people.

The mechanisms by which asbestos produces malignancy are not entirely clear. Various *in vitro* and *in vivo* studies have suggested that fiber dimension, surface properties, and physical durability are important criteria for the carcinogenicity of the fibers (*2,7*). Although reactive oxygen species (ROS) have been indicated as one of the key determinants of asbestos-induced mutagenesis and carcinogenesis, the types and origin of these free radicals remain elusive (*8,9*). It has been shown that ROS such as superoxide anions (O_2^-) and hydrogen peroxide originate not only from redox reactions catalyzed on the fiber surface but also from the incomplete phagocytosis of fibers in various cells, such as phagocytic, mesothelial, and rat lung epithelium cells (*10–12*). The involvement of ROS and the protective effects of antioxidants such as catalase and superoxide dismutase and radical scavengers such as dimethyl sulfoxide (DMSO) and Tempol in asbestos-induced toxicity have been studied in various cell systems as well (*8,13,14*). These biologically reactive ROS, in particular hydroxyl radicals (OH•), act directly or indirectly to damage neighboring biomolecules, such as DNA and membrane lipid (*14,15*). There is evidence to suggest that the array of DNA and chromosomal damages induced by ROS such as base substitutions, deletions, rearrangements, insertions, sister chromatid exchanges, and chromosomal aberrations may lead to a broad spectrum of mutations in mammalian cells (*16*). However, earlier studies on the mutagenicity of asbestos at either the *hprt* or *oua* loci in a variety of mammalian cells have resulted in mostly negative findings (*17,18*). Subsequent studies have suggested that this could be a result of multilocus deletions induced predominantly by asbestos, which are not compatible with the survival of the mutants.

Several mutagenicity assays, which detect either large chromosomal mutations, homologous recombination, or score mutants located on nonessential genes, have demonstrated the mutagenic potential of various fiber types (*19–22*). Although these findings have indicated a close relationship between chromosomal abnormalities that have frequently been shown in fiber-exposed human and rodent cell lines and carcinogenicity *in vivo*, there is less direct evidence to illustrate how ROS are involved in those processes.

The human–hamster hybrid (A_L) cell, which contains a full set of hamster chromosomes and a single copy of human chromosome 11, is sensitive in detecting mutagens that induce mostly large, multilocus deletions such as ionizing radiation and certain heavy metals (*23,24*). Because only a small part of region 11p15.5 is required for the viability of A_L cells, mutations in the human chromosome 11 ranging in size up to 140 Mbp of DNA can be detected. Compared with the hamster *hprt* locus, previous studies have shown that there is a 50-fold increase in mutant yield at the *CD59* locus in crocidolite-treated A_L cells (*25*).

In the present studies we focused on clarifying the role of ROS in mediating crocidolite-induced mutagenicity in mammalian cells. We followed the generation of ROS with the radical probe 5′,6′-chloromethy-2′,7′-dichlorodihydrofluorescein diacetate

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(CM-H_2DCFDA) in fiber-exposed live cells with or without DMSO. Increase in ROS production was associated with the oxidation of the nonfluorescent dye to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) (*26*). We further determined the formation of H_2O_2 from crocidolite fiber-treated cells using Amplex Red reagent either in the presence or absence of catalase. Because H_2O_2 can react with intracellular metals to produce ROS via the Fenton reaction, we speculated that asbestos fibers would induce similar types of mutations as that of chemically generated ROS (*27,28*). We found that there was a dose-dependent formation of ROS in crocidolite fiber-exposed A_L cells, and the protective effects of antioxidants were demonstrated by DMSO and catalase. Furthermore, we found that asbestos fibers were mutagenic and the types of mutants induced were similar to those of chemically generated ROS analyzed at two equitoxic doses. These results provide direct evidence that the genotoxicity of asbestos fibers is mediated by ROS.

Materials and Methods

Cell culture. We used the human–hamster hybrid (A_L) cell line containing a standard set of CHO-K1 chromosomes, and a single copy of human chromosome 11. Chromosome 11 contains the *CD59* gene (also known as *M1C1*) at 11p13.5, which encodes the CD59 cell-surface antigen marker (formerly known as S1) that renders A_L cells sensitive to killing by the monoclonal antibodies E7 in the presence of rabbit serum complement (HPR, Denver, PA). Antibody specific to the CD59 antigen was produced from hybridoma culture. Cells were cultured in Ham's F-12 medium supplemented with 8% heat-inactivated fetal bovine serum (Atlanta Biological, Norcross, GA), 2×10^{-4} M glycine, and 25 μg/mL gentamycin at 37°C in a humidified 5% CO_2/95% air incubator, and passaged as described by Hei et al. (*19,24,25*).

Preparation of crocidolite fibers. We used International Union Against Cancer standard reference crocidolite fibers (average length 3.2 ± 1.0 μm; average diameter 0.22 ± 0.01 μm) in these studies. The fibers were prepared as described previously (*8,25*). Briefly, samples of fibers were weighed and suspended in distilled water. The fiber suspension was triturated six to eight times with a 20-gauge syringe needle. A stock solution of the fibers was sterilized by autoclaving and mixed to ensure a uniform suspension before being diluted with tissue culture medium for cell treatment.

Treatment with antioxidants. Catalase (Sigma Chemicals, St. Louis, MO) was prepared fresh each time due to its unstable nature in aqueous solution (*8*). Stock catalase solution was membrane-filtered and diluted with medium to a working concentration of 1,000 U/mL. DMSO (Sigma) was diluted directly from stock solution with medium to a final concentration of 0.5% (v/v). To demonstrate the involvement of ROS in asbestos mutagenesis, exponentially growing A_L cells were exposed to asbestos for 24 hr with or without concurrent treatment with either catalase or DMSO. To ascertain the mutagenicity of H_2O_2 (30%; Sigma), we exposed exponentially growing A_L cells to H_2O_2 in serum-free medium for 15 min with or without concurrent treatment with either catalase or DMSO. The doses of catalase and DMSO used here were nontoxic and nonmutagenic. After treatment, cells were trypsinized and replated for survival and mutation assays.

Determination of intracellular ROS. Exponentially growing A_L cells were plated onto 35-mm glass-bottom microwell dishes (Bioptech Inc., Butler, PA). After overnight incubation, cells were pretreated with a 1-μM dose of the nonfluorescent, membrane-permeable dye CM-H_2DCFDA (Molecular Probes, Eugene, OR) at 37°C for 40 min as described by Long et al. (*26*). Subsequently, the cells were washed twice with cold ACAS buffer (127 mM NaCl, 0.8 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 4.4 mM $C_6H_{12}O_6$, 10 mM HEPES, pH 7.4) to decrease metabolic activity and remove any excess dye. We added 1 ml ACAS buffer or fiber suspension with or without DMSO to the dishes, which was rapidly warmed to 37°C on the Zeiss Axiovert 100 TV microscope (Carl Zeiss, Thornwood, NY). Cells were viewed by using 100 × 1.4 objective lens equipped with a laser scanning confocal attachment (model LSM410, Zeiss). CM-H_2DCFDA was excited with the 488-nm line of an argon/krypton mixed gas laser. Emission was collected with a 510-nm long pass filter. A semiquantitative estimation of ROS-associated fluorescent signals was obtained using the composite images generated by Adobe Photoshop (Adobe Systems, Mountain View, CA). We randomly selected 60–80 individual cells per dose per experiment and quantified the fluorescent images as described by Liu et al. (*29*). On average, we measured up to 200 cells per exposed group.

Fluorescent microassay of hydrogen peroxide. We measured the release of H_2O_2 by horseradish peroxidase (HRP)-dependent oxidation of *N*-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent; Molecular Probes) in 96-well tissue culture plates as described by Mohanty et al. (*30*). Briefly, 100 μl Amplex Red reagent solution (144.4 mM NaCl, 5.7 mM Na_3PO_4, 4.86 mM KCl, 0.54 mM $CaCl_2$, 1.22 mM $MgSO_4$, 75 μM Amplex Red reagent, pH 7.35) containing 0.5 U/mL HRP (Molecular Probes) and graded doses of crocidolite fibers in the presence or absence of 1,000 U/mL catalase were added to 96-well microplates and incubated at 37°C for 15 min. We replated 20 μl of exponentially growing A_L cells at a density of 2×10^6/mL into the microplates and incubated them at 37°C for 4 hr. We measured the fluorescence intensity of each well using a microplate reader (Bio-Tek Instruments, Inc., Winooski, VT) with an excitation wavelength in the range of 530–560nm. The concentration of H_2O_2 was determined based on a standard curve.

Cytotoxicity of crocidolite fibers and hydrogen peroxide. Exponentially growing A_L cells were treated with graded doses of fibers for 24 hr or with H_2O_2 in serum-free medium for 15 min. After treatment, cultures were washed with balance salt solution, trypsinized, and replated into 100-mm diameter Petri dishes for colony formation. The cultures were incubated for 7 days, at which time they were fixed with formaldehyde and stained with Giemsa. We counted the number of colonies to determine the surviving fraction as described (*19,25*).

Quantification of mutations at the* CD59 *locus. After completion of the various treatments, the cultures were replated into T75 flasks and cultured for 1 week before mutagenesis testing began as described (*23,25*). This expression period is necessary for the surviving cells to recover from the temporary growth lag induced by either crocidolite fibers or H_2O_2 and multiply sufficiently, so that the progeny of the mutated cells no longer express lethal amounts of the CD59 surface antigen. To determine mutant fraction, 5×10^4 cells were plated into each of six 60-mm dishes in a total of 2 mL of growth medium. After incubation for 2 hr, we treated the cultures with 0.2% CD59 antiserum and 1.5% (v/v) freshly thawed complement. Controls were composed of identical sites of dishes including antiserum alone, complement alone, or neither agent. The cultures were incubated for 7–10 days, at which time they were fixed, stained, and the surviving colonies were scored. We tested the colonies for mutant yield each week for 2 consecutive weeks to ensure the full expression of mutations. We calculated mutant fractions as the number of surviving colonies divided by the total number of cells plated after correction for any nonspecific killing due to complement alone.

Analyses of mutant spectrum by multiplex polymerase chain reaction. *CD59*$^-$ mutants were isolated by cloning and expanded in culture as described (*24*). To ensure that all mutants analyzed were independently generated, we isolated only one and, occasionally, no more than two well-separated mutants per dish for analysis. Five marker genes located on either the short arm (*WT, PTH, CAT, RAS*) or the long arm (*APO-A1*) of human chromosome 11 were chosen for multiplex polymerase chain reaction (PCR) because of their mapping

positions relative to the *CD59* gene and the availability of PCR primers for the coding regions of these genes. PCR amplifications were performed for 30 cycles using a DNA thermal cycle model 480 (Perkin-Elmer/Cetus) in 20-µl reaction mixtures containing 0.2 µg of the *Eco*RI-digested DNA sample in 1× Stoffel fragment buffer, all four dNTPs (each at 0.2 mM), 3 mM $MgCl_2$, 0.2 mM each primer, and 2 U Stoffel fragment enzyme. Each PCR cycle consisted of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. After the last cycle, samples were incubated at 72°C for an additional 20 min, electrophoresed on 3% agarose gels, and stained with ethidium bromide.

Statistics. We analyzed data using Student's *t*-tests. Differences between means were regarded as significant if $p < 0.05$.

Results

Intracellular ROS production induced by crocidolite fibers. If generation of ROS is one of the major mechanisms for asbestos-induced mutagenesis in mammalian cells, then fiber treatment should be expected to induce ROS production in the A_L cells. To quantify intercellular ROS induced by crocidolite fibers, A_L cells were pretreated with CM-H_2DCFDA, which passively diffused into cells and was oxidized by ROS to a fluorescent form (*29*). Figure 1A and C illustrate CM-H_2DCFDA fluorescent imaging in control A_L cells and cells treated with a 6 µg/cm^2 dose of crocidolite fibers, respectively. These images show a typical field of the various treatment groups, generated from composite confocal images of 11 sagittal sections. Cells exposed to fibers exhibited a higher fluorescence level when compared to the control, indicative of higher intercellular oxidant levels. However, some fluorescence was detected in the control culture, which might be due to the normal oxidative metabolism of cells such as mitochondrial respiration.

A dose-dependent induction of ROS in A_L cells treated with asbestos fibers is shown in Figure 2. Quantification of relative fluorescence in fiber-treated and control cells indicated that treatment of cells with a 6 µg/cm^2 dose of crocidolite fibers induced a 5-fold increase in the generation of ROS compared to the control ($p < 0.05$). However, there was no further increase in fluorescence induction with fiber concentration > 6 µg/cm^2. The oxyradical nature behind the increase in fluorescence intensity was further supported by including the radical scavenger DMSO in the reaction mixture. Although DMSO alone had little effect on the formation of ROS among control cells (Figure 3), the relative fluorescence level induced by a 6 µg/cm^2 dose of fibers in A_L cells decreased from 59.01 ± 6.74 to 19.30 ± 0.85 in the presence of DMSO ($p < 0.05$), which was consistent with our previous studies of a suppressive effect of DMSO on the formation of 8-hydroxy-deoxyguanosine in crocidolite-treated A_L cells (*31*).

Hydrogen peroxide production induced by crocidolite fibers. Figure 4 shows the release of H_2O_2 from asbestos-treated A_L cells based on HRP-catalyzed oxidation of fluorescent Amplex Red reagent in the presence or absence of catalase. There was a dose-dependent induction of H_2O_2, reaching a peak of 0.32 ± 0.055µM at a 6 µg/cm^2 dose of fibers. Catalase, which directly metabolizes H_2O_2 to water and oxygen, has been shown to be an effective free-radical scavenger in various cell systems (*32*). Concurrent treatment of A_L cells with crocidolite fibers at a dose of 6 µg/cm^2 and catalase (1,000 U/mL) suppressed H_2O_2 induction by 7- to 8-fold ($p < 0.05$). The dose of catalase used here had little effect on the level of H_2O_2 in control cells. Likewise, heat-inactivated catalase (by boiling for 10 min) had little effect on the fluorescence intensity of fiber-treated A_L cells.

Cytotoxicity and mutagenicity of crocidolite fibers and hydrogen peroxide. To show that ROS induced by asbestos fibers actually mediate the mutagenic events, it is necessary

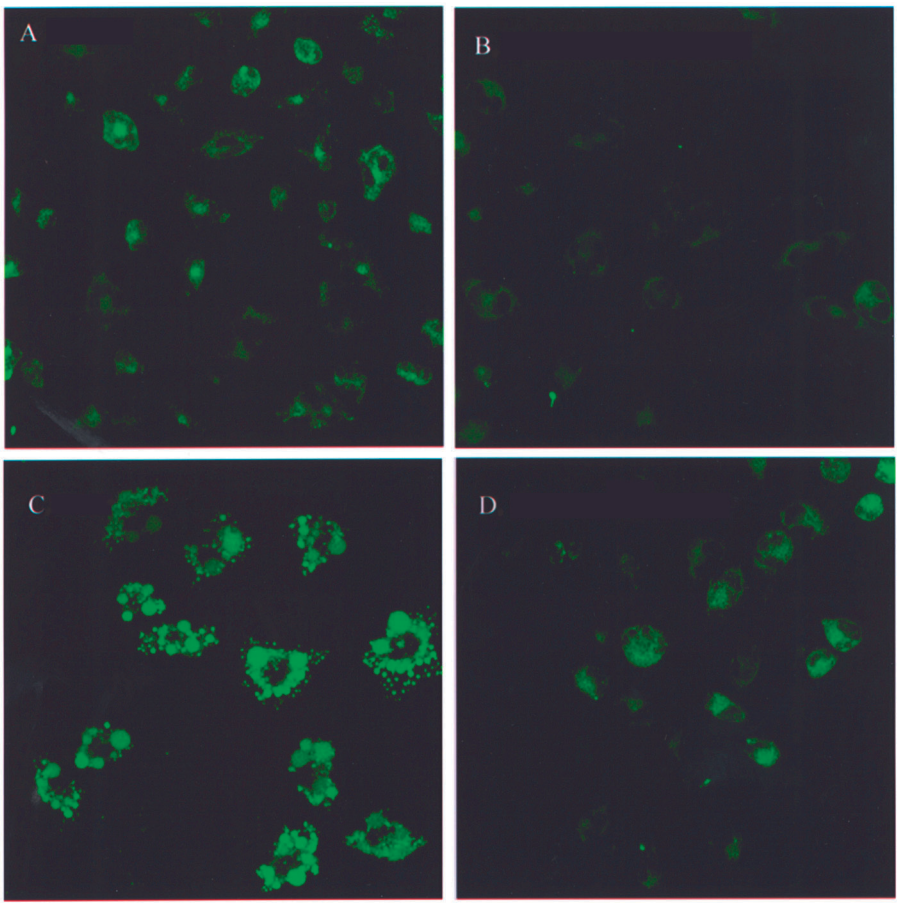

Figure 1. Representative image of fluorescent signals generated from composite images obtained by confocal microscopy of A_L cells pretreated with the radical probe CM-H_2DCFDA for 40 min with or without subsequent asbestos treatment. (*A*) Control A_L cells treated with only fluorescent probe; (*B*) control cells in the presence of 0.5% DMSO; (*C*) 20 min after treatment with a 6 µg/cm^2 dose of crocidolite fibers; (*D*) treatment with crocidolite and concurrent 0.5% DMSO (600×).

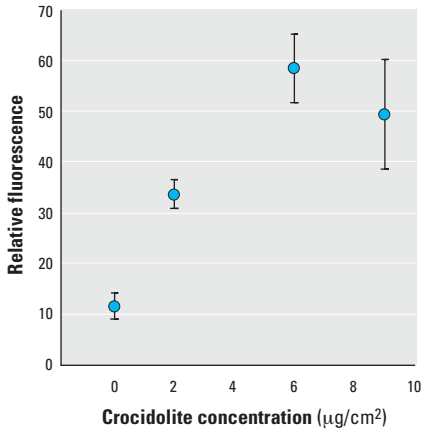

Figure 2. Relative fluorescence intensity of ROS in A_L cells as a function of crocidolite concentration. Exponentially growing A_L cells were exposed to graded doses of crocidolite, and ROS were determined by CM-H_2DCFDA fluorescence staining. The relative intensities are expressed in arbitrary units. Data were pooled from three independent experiments. Error bars indicate SD.

to demonstrate that chemically generated ROS is mutagenic and induces a similar spectrum of mutants as that of asbestos fibers. Exposure of A_L cells to either graded doses of crocidolite fibers for 24 hr or H_2O_2 in serum-free medium for 15 min resulted in a dose-dependent increase in toxicity of A_L cells (Figure 5). The normal plating efficiency of A_L cells was 80 ± 5% in the present studies. The surviving fraction of A_L cells treated with a 2 μg/cm^2 dose of crocidolite fibers was 62 ± 4%, and the value decreased to 26 ± 5% after treatment with a 4 μg/cm^2 dose of crocidolite fibers. By comparison, survival of A_L cells after exposing to 4.4 mM or 13.2 mM H_2O_2 was 56 ± 10% and 24 ± 5%, respectively. The background mutant fraction of A_L cells used in these experiments averaged 52 ± 15 mutants per 10^5 survivors. In contrast, the negative control, titanium dioxide (TiO_2), at doses up to 12 μg/cm^2 was neither cytotoxic nor mutagenic to A_L cells when tested under similar conditions (data not shown). Both crocidolite fibers and H_2O_2 led to a dose-dependent induction of *CD59*$^-$ mutants in A_L cells. The mutant fraction increased with the doses of fibers and reached a level that was approximately 4-fold higher than background at a 4 μg/cm^2 dose of fibers in A_L cells. As shown in Figure 5, the mutant fraction was slightly higher among cells treated with H_2O_2 than those exposed to crocidolite fibers at equally toxic doses, though the difference was not statistically significant. Furthermore, the mutation yields induced by either crocidolite fibers at a dose of 4 μg/cm^2 or 13.2 mM H_2O_2 were dramatically suppressed by either 0.5% DMSO or 1,000 U/mL catalase ($p < 0.05$; Figure 6). These results confirm that ROS plays a casual role in the mutagenicity of crocidolite fibers and H_2O_2.

Analysis of mutant spectra. To compare the type and size of mutations that caused the CD59 phenotype among A_L cells exposed to either crocidolite fibers or H_2O_2, multiplex PCR and primer sequences for five marker genes (*WT*, *PTH*, *CAT*, *RAS*, and *APO-A1*) located on either the short or long arm of human chromosome 11 were used as described (*24*). These primers and PCR conditions were selected to amplify only the human genes instead of their CHO cognate. Because there is only one chromosome 11 in A_L cells, the presence or absence of the corresponding PCR products indicates that a particular segment of DNA containing these genes is present or lost, respectively. There is evidence to suggest that a small region of the distal end of human chromosome 11 at 11p15.5, which corresponds to the RAS probe in all mutants, is required for viability of the hybrid cells (*33,34*). As shown in Table 1, the majority of spontaneous *CD59*$^-$ mutants (22/30 or 73%) showed no detectable changes in any of the marker genes examined, which was consistent with previous studies (*24,34*). In contrast, only 9/27 or 33% of mutants derived from cells exposed to a 2 μg/cm^2 dose of fibers retained all of the marker genes examined, and 18/27 or 78% of the mutants had lost at least one additional marker, which included 7/27 or 26% that lost the proximal APO-A1 located on the long arm of the chromosome as well. The proportion of mutants suffering loss of additional chromosomal markers increased with increasing concentration of fibers such that none of the 29 mutants induced by a 4 μg/cm^2 dose of fibers retained all five of the marker genes, and 19/29 or 66% of them lost the long arm marker in addition to the *CAT* and *WT* gene on the short arm of human chromosomal 11. The types of mutants induced were similar to those induced by an equivalent cytotoxic dose of H_2O_2 in that 9/23 or 39% of the mutants induced by a 4.4 mM H_2O_2 retained all five primers compared to none among those induced by the higher dose of 13.2 mM. These results indicated that equitoxic doses of crocidolite fibers and H_2O_2 induced similar multilocus deletions that were increased in a dose-dependent manner, providing strong circumstantial evidence that similar mutagenic mechanisms are involved in the mutagenicity induced by fibers and ROS.

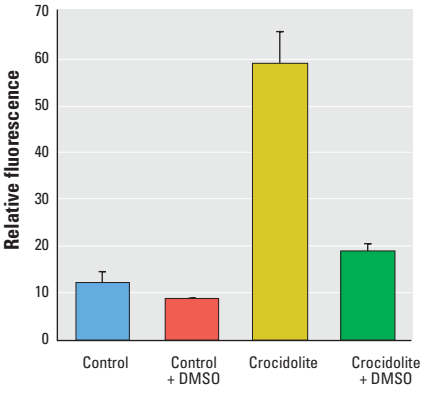

Figure 3. Effect of the radical scavenger DMSO (0.5%) on the induction of ROS in A_L cells treated with a 6-μg/cm^2 dose of crocidolite. The relative intensities were expressed in arbitrary units. Data were pooled from three independent experiments. Error bars indicate SD.

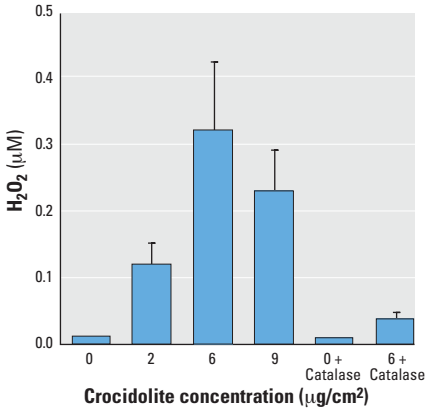

Figure 4. Induction of H_2O_2 in A_L cells exposed to graded doses of crocidolite fibers either in the presence or absence of catalase at a concentration of 1,000 U/mL. Exponentially growing A_L cells were plated in 96-microwell plates, and the release of H_2O_2 was examined using Amplex Red reagent in the presence of HRP. Data were pooled from three independent experiments. Error bars indicate SD.

Discussion

The mechanisms by which ROS are generated in response to asbestos fiber exposure have been widely studied (*10*). Recent studies have shown that asbestos-induced toxicity to hamster tracheal epthelial cells, rat lung fibroblasts, and rat alveolar macrophages is suppressed by the antioxidant enzymes, superoxide dismutase, and catalase, as well as the free-radical scavengers, such as cysteine, dimethylthiourea, and ascorbic acid (*11,12,36,37*). The finding that mesothelioma induction in rats and humans can be correlated with fiber-induced hydroxyl radicals provides further support for the possible role of ROS in fiber carcinogenesis (*38*). Although crocidolite, the most carcinogenic type of asbestos, has been shown to catalyze the formation of hydroxyl radicals by either Fenton or Haber-Weiss reactions and induces lipid peroxidation, DNA strand breaks, sister chromatid exchanges, and clastogenicity (*14,39,40*), its carcinogenic and mutagenic mechanisms are still poorly understood.

Several methods, including reduction of cytochrome c by superoxide ion, luminal chemoluminescence, reduction of scopoletin emission, and electron spin resonance, have been used to measure the generation of ROS

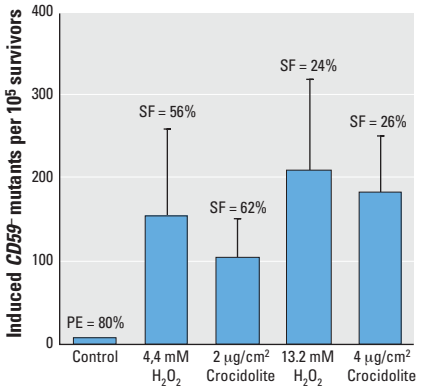

Figure 5. Induction of *CD59*$^-$ mutant fractions per 10^5 survivors in A_L cells exposed to either crocidolite fibers or H_2O_2. Abbreviations: PE, plating efficiency; SF, surviving fraction. Results are expressed as number of induced mutants (total mutant yield minus background) per 10^5 survivors. The average number of preexisting *CD59*$^-$ mutants per 10^5 survivors in A_L cells used for these experiments was 52 ± 15. Data were pooled from three independent experiments. Error bars indicate SD.

induced by asbestos, but such assays are typically performed in large samples of cells (~10^6) and are affected by geometry and number of fibers (*41–43*). An ideal technique would be to measure ROS released as single-cell interaction with defined doses of asbestos. CM-H_2DCFDA is a well-established free-radical probe, which can be trapped in live cells and provides reliable intracellular fluorescent signals (*44,45*). The oxidation of CM-H_2DCFDA by ROS, as detected using confocal microscopy, provided strong evidence that asbestos induced a dose-dependent increase of ROS in single cells, which could be inhibited by DMSO.

Although the present data clearly demonstrate the induction of ROS in fiber-induced mutagenesis in A_L cells, these experiments did not specifically identify the source of these radical species. It is generally accepted that the mitochondrion is the major source of intracellular ROS, which is enhanced by electron transport inhibitors such as ischemia-reperfusion, rotenone, antimycin A, and diphenyleneiodonium (*46–48*). It is possible that the mitochondrial membrane damage induced by asbestos could trigger a cascading event in ROS production involving lipid peroxidation (*49*). Alternatively, peroxynitrite anions generated as a result of mitochondrial damage could also be involved. Recent evidence has indicated that stimulation of an NADH or NADPH oxidase and/or conversion of xanthine dehydrogenase to xanthine in the cytoplasm can occur after contact of fibers with the plasma membrane and their subsequent uptake by the cells (*32,50*). We observed that ROS were localized mainly in the cytoplasm, especially in spherical organelles, both in the control and fiber-treated cells pretreated with CM-H_2DCFDA.

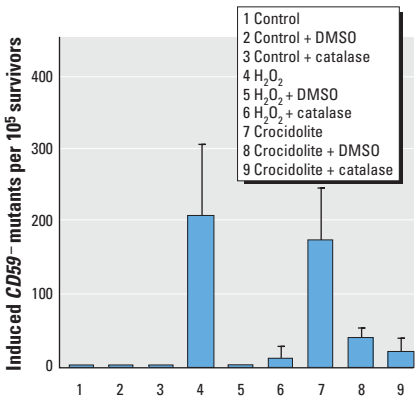

Figure 6. Effects of catalase (1,000 U/mL) and DMSO (0.5%) on induced *CD59*⁻ mutant fraction per 10^5 survivors in A_L cells exposed to either crocidolite (4 μg/cm^2) or H_2O_2 (13.2 mM). Data were pooled from four independent experiments. Error bars indicate SD.

Among those ROS induced by asbestos fibers, H_2O_2 is relatively long lived and directly crosses cell membranes by simple diffusion (*42*). There is evidence that H_2O_2 induces not only damage to DNA, causing single- and double-strand breaks, base loss, base substitution, and cross-linking, but that it also causes chromosome aberrations, as well as chromatid aberrations (*47*). Using the spin trap 5,5′-dimethyl-1-pyrroline-*N*-oxide (DMPO), Weitzman and Graceffa (*27*) demonstrated that crocidolite fibers catalyze the production of OH$^\bullet$ from H_2O_2 in a cell-free system. In this study, we examined the role of H_2O_2 generation in A_L cells exposed to crocidolite fibers in the presence or absence of catalase. Our data suggested that H_2O_2 may be an important mediating molecule, which is responsible for fiber cytotoxicity. Because catalase is a relatively large molecule (molecular weight of 250 kD), it is highly unlikely to pass across the cell membrane without being phagocytized. On the other hand, H_2O_2 is freely diffusible between intracellular and extracellular space, and addition of extracellular catalase can reduce the intracellular oxidative stress induced by fibers. In addition to the well-documented iron-catalyzed reactions that generate a variety of ROS such as O_2^-, H_2O_2, OH$^\bullet$, $^1O_2$, $HO_2^\bullet$, and lipid peroxy radicals, another pathway involving reactive nitrogen species may be involved. Several studies have shown that exposure to crocidolite increases the production of nitric oxide in rat macrophages and human lung epithelial (A549) cells, which subsequently reacts with $O_2^\bullet$ to produce OH$^\bullet$ and peroxynitrite ($ONOO^-$) (*51,52*). The latter has been shown to cause nitration of proteins, hydroxylation or nitration of DNA, and mutations (*53*).

Previous studies have suggested that asbestos fibers are strong gene and chromosomal mutagens, inducing predominantly large deletions in A_L cells (*8,19,25*). To obtain further insight into the role of oxidative DNA and chromosome damage in asbestos-mediated carcinogenesis, it is necessary to compare the mutation pattern between crocidolite fibers and ROS. In the absence of serum, H_2O_2 produced predominantly OH$^\bullet$ radicals in human fibroblast culture (*37*). Using this approach, we show that in asbestos-induced *CD59*⁻ mutants, the types of mutation induced are similar to those induced by H_2O_2. From a mechanistic point of view, these data suggest that similar mutagenic mechanisms are involved between asbestos fibers and chemically generated ROS, which provides further evidence that fiber-induced mutagenesis may be mediated through ROS.

Because most ROS are short-lived and can only diffuse short distances in cells, it is still not clear how these radicals reach the nucleus to cause gene and chromosomal mutation (*54,55*). Compared with the parental Chinese hamster ovary cells, A_L cells contain similar levels of glutathione peroxidase, superoxide dismutase, and total glutathione, but a 50% increase in catalase. This is due, presumably, to the extra copy of *CAT* gene from the single copy of human chromosome 11 that they contain (*56*). One possible scenario is that free radicals generated by asbestos fibers lead to a cascading event involving lipid peroxidation. A direct correlation between lipid peroxidation and fiber mutagenesis/carcinogenesis has not been demonstrated, although there is evidence that asbestos fibers induce lipid peroxidation in bacteria and mammalian cells (*49,57*).

Table 1. The number (percent) of *CD59*⁻ mutants either of spontaneous origin or induced by the various treatments that retain the following markers in human chromosome 11 as determined by multiplex PCR analyses.

Group	Total mutants	Marker				
		APO-A1	CAT	WT	PTH	RAS
Spontaneous	30	30 (100)	24 (80)	22 (73)	30 (100)	30 (100)
2 μg/mL Crocidolite	27	20 (74)	16 (59)	9 (33)	27 (100)	27 (100)
4 μg/mL Crocidolite	29	10 (34)	11 (38)	0 (0)	29 (100)	29 (100)
4.4 mM H_2O_2	23	17 (74)	10 (43)	9 (39)	23 (100)	23 (100)
13.2 mM H_2O_2	31	14 (45)	8 (26)	0 (0)	31 (100)	31 (100)

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